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Regulation of Sesquiterpene Cyclase Gene Expression[1]

Characterization of an Elicitor- and Pathogen-Inducible Promoter

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The promoter for a tobacco (*Nicotiana tabacum*) sesquiterpene cyclase gene, a key regulatory step in sesquiterpene phytoalexin biosynthesis, has been analyzed. The EAS4 promoter was fused to the β-glucuronidase (GUS) reporter gene, and the temporal and spatial expression patterns of GUS activity were examined in stably transformed plants and in transient expression assays using electroporated protoplasts of tobacco. No GUS activity was observed in any tissues under normal growth conditions. A low level of GUS activity was detected in wounded leaf, root, and stem tissues, whereas a much higher level was observed when these tissues were challenged with elicitors or microbial pathogens. The GUS expression pattern directed by the EAS4 promoter was identical to the induction patterns observed for the endogenous sesquiterpene cyclase genes. Neither exogenous salicylic acid nor methyl jasmonate induced GUS expression; and H_2O_2 induced GUS expression to only a limited extent. Although the EAS4 promoter contains *cis*-sequences resembling previously identified transcriptional control motifs, other *cis*-sequences important for quantitative and qualitative gene expression were identified by deletion and gain-of-function analyses. The EAS4 promoter differs from previously described pathogen-/elicitor-inducible promoters because it only supports inducible gene expression and directs unique spatial expression patterns.

One of the most extensively studied responses of plants to pathogen attack and other abiotic stresses is the synthesis and accumulation of antimicrobial phytoalexins (Dixon, 1986; Ku'c, 1995). Synthesis and accumulation of phenylpropanoid-derived compounds, for example, are well documented for French bean (Dixon et al., 1983; Dixon and Harrison, 1990), alfalfa (Paiva et al., 1991), and parsley (Hahlbrock et al., 1981; Hahlbrock and Scheel, 1989) cells and tissues responding to a variety of stress inducers. In contrast, bicyclic sesquiterpene phytoalexins accumulate in cell cultures and tissues of tobacco (*Nicotiana tabacum*) and *Capsicum annuum* challenged with pathogen-derived elicitors such as cellulase, fungal cell wall hydrolysates, and cryptogein, an extracellular protein produced by *Phytophthora cryptogea* (Brooks et al., 1986; Vögeli and Chappell, 1988; Ricci et al., 1989; Whitehead et al., 1989; Milat et al., 1991). Sesquiterpene phytoalexins are characteristic of the Solanaceae and are 15-C derivatives arising from the isoprenoid biosynthetic pathway (Chappell, 1995; Ku'c, 1995). Similar to other classes of phytoalexins in other plant families, sesquiterpene phytoalexins are not found in healthy or control tissues, but only accumulate in response to elicitation or pathogen challenge (Bailey et al., 1975; Watson and Brooks, 1984; Brooks et al., 1986; Chappell and Nable, 1987).

Earlier work demonstrated that a key regulatory step in the biosynthesis of capsidiol, the principal sesquiterpene phytoalexin found in tobacco and pepper, is catalyzed by EAS (Vögeli and Chappell, 1988; Facchini and Chappell, 1992). EAS is a sesquiterpene cyclase located at a putative branch point in the isoprenoid biosynthetic pathway and is likely responsible for the diversion of C from the general isoprenoid pathway to phytoalexin biosynthesis (Cane, 1981; Vögeli and Chappell, 1988). In tobacco, sesquiterpene cyclase enzyme activity is absent from control or noninduced cell cultures, but is induced to high levels within 8 to 12 h of elicitor treatment. Previous studies have also demonstrated that the induction of this enzyme requires the de novo synthesis of the enzyme protein as well as the transcriptional activation of the corresponding gene(s) (Vögeli and Chappell, 1990; Facchini and Chappell, 1992). Together, these results suggest that EAS is an important control point for the synthesis of sesquiterpene phytoalexins, and that the level of this enzyme's activity is primarily under transcriptional control.

Facchini and Chappell (1992) previously reported 12 to 15 copies of the sesquiterpene cyclase gene within the tobacco genome, suggesting a complex gene family wherein individual genes may be differentially regulated during development or in response to environmental stim-

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Abbreviations: CaMV, cauliflower mosaic virus; CHS, chalcone synthase; EAS, 5-epi-aristolochene synthase; IFR, isoflavone reductase; MJ, methyl jasmonate; PAL, Phe ammonia-lyase; SA, salicylic acid; SAR, systemic acquired resistance.

uli. Two particular members of this gene family have been cloned and sequenced. These genes, referred to as *EAS3* and *EAS4*, are found as a tandem repeat within 10 kb of each other. However, despite a very high homology (>90%) within the coding regions of these genes, they are divergent in their 5′ and 3′ sequences, which appear in mRNA as untranslated sequences. Based on finding 3′ nontranslated sequences in a cDNA clone identical to those in *EAS4*, Back et al. (1994) concluded that expression of the *EAS4* gene was elicitor inducible. No EAS cDNA clone containing 5′ or 3′ sequences homologous to those specific for the EAS3 genomic clone has yet been found.

The elicitor-induced activation of sesquiterpene cyclase genes occurs very rapidly. For example, RNA isolated from 2-h cellulase-treated cell cultures already contained a significant amount of translationally active cyclase mRNA (Vögeli and Chappell, 1990). A similar rapid induction has also been observed for PAL and CHS, key enzymes of phenylpropanoid phytoalexin biosynthesis in bean (Dixon et al., 1983; Liang et al., 1989), and alfalfa (Jorrin and Dixon, 1990), and has been further documented to result from a more rapid increase in the transcription rate of the respective gene (Cramer et al., 1985; Somssich et al., 1986; Lawton and Lamb, 1987). Such results suggest that the rapid and selective transcriptional activation of defense gene expression must be coupled to an elicitor recognition event by a very efficient signal transduction cascade (Lamb et al., 1989; Hahlbrock et al., 1995). Little, however, is known about the signal transduction pathways involved in these processes.

SA, MJ, and H_2O_2 have been suggested as possible signal molecules mediating plant defense gene expression and phytoalexin accumulation (Farmer and Ryan, 1992; Gundlach et al., 1992; Chen et al., 1993; Delaney et al., 1994; Levine et al., 1994; Mehdy, 1994; Reinbothe et al., 1994; Ryals et al., 1995), but a role for these compounds in the activation of sesquiterpene cyclase gene expression and in the accumulation of sesquiterpene phytoalexins has not been fully explored.

Although cell cultures are simple and convenient experimental systems for studying the regulation of sesquiterpene cyclase genes, they cannot provide direct information on the temporal and spatial pattern of gene activation within an intact plant or in response to direct pathogen challenge. In the current work the regulatory properties of the EAS4 promoter are examined by analyzing GUS activity in transgenic tobacco plants harboring EAS4 promoter-GUS gene constructs. The sensitive GUS assay has allowed us to detect elicitor- or pathogen-induced transcriptional activity of the EAS4 promoter at the cellular level, and was used to examine cell- and tissue-specific expression in intact plants. Our data demonstrate that the EAS4 promoter contains *cis*-acting elements capable of conferring qualitative and quantitative expression patterns that are exclusively associated with pathogen-, elicitor-, or wound-induction patterns. A possible role of SA, MJ, and H_2O_2 as regulators of *EAS4*-specific expression or expression of other endogenous sesquiterpene cyclase genes has also been examined.

MATERIALS AND METHODS

Elicitor, Pathogen, and Chemical Treatments

Cryptogein was prepared according to the method of Ricci (1989), and was kindly provided by Dr. Yu (University of California, Davis). Approximately 50-μL aliquots of water (control) or elicitor (cryptogein, 25 nM) were infiltrated into fully expanded leaves from the abaxial side with a repeat pipetter (Eppendorf). The infiltrated tissues were collected using a cork borer 2 cm in diameter at the indicated times and were frozen at −80°C until they were analyzed for GUS activity. Cryptogein was also used to elicit root and stem segments. Roots were obtained from tobacco (*Nicotiana tabacum*) plants maintained on solid Murashige and Skoog medium, and upper portions of stems (4 cm below the apex) were from 2-month-old plants grown in a greenhouse. Roots and stems were cut into 0.5-cm segments and incubated with water or 100 nM cryptogein at room temperature under sterile conditions.

Pseudomonas syringae pv *syringae* 61 and its *hrpH* mutant were a gift from Dr. He (Plant Research Laboratory, Michigan State University). The *P. syringae* cells were grown overnight in Luria Bertani medium, washed twice with water, and resuspended in sterile water. Approximately 50-μL aliquots of water (control) or bacterial suspensions (A_{600} = 0.05) were locally infiltrated into leaves.

Young, apical leaves were detached from 2-month-old transgenic tobacco plants and inoculated with two to four mycelia plugs (1 cm in diameter) of 2-d-old *Phytophthora parasitica* var *nicotianae* cultures (race 0 and race 1) grown on oatmeal agar (Tedford et al., 1990). Inoculated leaves were incubated on filter paper moistened with distilled water in a growth chamber at 25°C with constant fluorescent lighting. Control samples were inoculated with sterile agar plugs. Inoculated tissues were collected for GUS assay at the indicated times using a cork borer 2 cm in diameter.

For chemical treatments leaf discs were floated on water containing sodium salicylate (Sigma), MJ (kindly provided by Dr. Hildebrand, University of Kentucky, Lexington), or H_2O_2 at the indicated concentrations in Petri dishes at room temperature for 24 h before tissue samples were collected for GUS assays.

Sesquiterpene Cyclase Enzyme Assay and Immunoblotting

Cyclase enzyme activity was measured as described previously (Vögeli and Chappell, 1990). Proteins were extracted from control and elicitor-treated tobacco tissues by homogenization with 80 mM KPO_4 buffer, pH 7.0, containing 20% (w/v) glycerol, 10 mM sodium metabisulfite, 10 mM sodium ascorbate, 15 mM $MgCl_2$, and 5 mM β-mercaptoethanol. Protein concentration was determined by the Bio-Rad assay. Equal quantities of protein (20–50 μg) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunodetected with a monoclonal antibody raised against purified tobacco sesquiterpene cyclase protein (Vögeli et al., 1990).

Gene Construction

A 1.9-kb *Hin*dIII-*Hin*dIII fragment of the tobacco genomic clone *gEAS4* containing a 1.2-kb 5′ untranslated flanking sequence was ligated into the polylinker of the pBluescript KS(+) vector (Facchini and Chappell, 1992) and sequenced according to the dideoxynucleotide chain-termination method (Sanger et al., 1977) with Sequenase 2.0 (United States Biochemical). The DNA sequence was verified by sequencing both strands of DNA. The *Hin*dIII-*Bam*HI fragments of the full-length *EAS4* promoter and its 5′ deletions were generated from this 1.9-kb pBluescript clone using PCR and synthetic oligonucleotides, and fused in frame with the *GUS* reporter gene in the pBI221 vector or the pBI101.1 binary vector (Clontech, San Diego, CA), respectively. The oligonucleotides were designed to have either a recognition sequence for *Hin*dIII (upstream primers) or *Bam*HI (downstream primer) plus 17 to 20 bp of the promoter sequence. All of these *Hin*dIII-*Bam*HI fragments have 3′ end points at +67 relative to the transcription start site. In all cases the 5′ end points of the deleted EAS4 promoter were confirmed by DNA sequencing.

Gain-of-function constructs were prepared by cloning the PCR fragments of the EAS4 promoter −266 to −87, −266 to −187, or −160 to −87 upstream of the CaMV 35S minimal promoter (−90–+8), which was fused to the GUS-coding region. The PCR fragments of the EAS4 promoter containing the *Pst*I/*Bst*EII restriction sites were blunt-end ligated into the *Eco*RV site (located at −90) of the 35S promoter, which was initially isolated from the pBI221 vector as a *Hin*dIII/*Bam*HI fragment and cloned in pBluescript KS(+). The chimeric promoter fragments with correct orientation were then isolated after digestion with *Pst*I and *Bam*HI and fused with the *GUS* reporter gene in pBI101.1. Junction sequences were confirmed by DNA sequencing.

Plant Transformation and Regeneration

Constructs cloned into binary vector pBI101.1 were transferred to the disarmed *Agrobacterium tumefaciens* strain GV3850 by the triparental mating procedure as described by Schardl et al. (1987). Tobacco plants (cv Xanthi) were transformed with *A. tumefaciens* harboring the various gene constructs by the standard leaf disc method, and kanamycin-resistant plants were regenerated as described by Horsch et al. (1985). Transgenic plants containing GUS driven by the CaMV 35S promoter were also generated as a means for comparing constitutive versus inducible expression. Seeds from regenerated (R_0) tobacco plants were germinated on medium containing 100 mg/L kanamycin, and the kanamycin-resistant plants were then grown in a greenhouse. Fully expanded leaves (7th and 8th from bottom) of 2-month-old plants were used for GUS analysis.

Protoplast Isolation and Electroporation

Protoplasts were isolated from suspension cells of 2-d-old cultures of *Nicotiana edwardsonii* as described by Kiernan et al. (1993). Suspension-cultured cells were collected by centrifugation and 1 volume of packed cells was resuspended in 2 volumes of cell wall digestion solution containing 1.5% cellulase (Onozuka RS, Karlan Chemical, Santa Rosa, CA), 0.15% pectolyase (Karlan Chemical) (w/v) in MMC medium (0.5 M mannitol, 10 mM Mes, and 10 mM $CaCl_2$, pH 5.6). The mixture was incubated at room temperature on a gyratory shaker for 3 to 4 h. Protoplasts (25-mL aliquots) were initially isolated by centrifugation at 100*g* for 90 s, then resuspended with a 25% Suc solution containing 10 mM Mes and 10 mM $CaCl_2$ up to a total volume of 12 mL. One milliliter of MMC medium was layered on top of the resuspended protoplast solution and the tubes were centrifuged immediately at 500*g* for 2 min. Purified protoplasts were removed with a pipette from the interface, diluted into 50 mL of MMC medium, and kept on ice for 3 h before electroporation.

Immediately prior to electroporation, aliquots containing 10^6 protoplasts were concentrated by centrifugation, resuspended in 1 mL of electroporation buffer (0.5 M mannitol, 5 mM Mes, and 70 mM KCl, pH 5.6), and mixed with 25 μg of plasmid DNA purified by CsCl centrifugation. Electroporation was performed using 125 V for 20 ms (Fromm et al., 1985). The transfected protoplasts were washed with 10 mL of MMC medium by centrifugation, resuspended in 8 mL of incubation medium (0.5 M mannitol, 10 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, 1 mM KI, 0.001 mM $CuSO_4$, and 1 μg/mL 2,4-D, pH 5.6), and incubated with or without further addition of 1 μg/mL cellulase (*Trichoderma viride*, Sigma). Protoplasts were incubated at room temperature under light for 18 h before measuring GUS activity.

Fluorometric GUS Assay

Intact plant tissues were homogenized with a mortar and pestle in extraction buffer containing 50 mM $NaPO_4$, pH 7.0, 0.05% Triton X-100, and 0.1% β-mercaptoethanol. Protoplasts were homogenized by vortexing in an Eppendorf tube. The homogenate was centrifuged for 5 min in a microcentrifuge, and the supernatant was collected for GUS assay. GUS activity in crude extracts was determined as described by Jefferson et al. (1987) using 4-methylumbelliferyl β-D-glucuronide as a substrate and 4-methylumbelliferone to calibrate the fluorometer. Protein content was determined according to the Bio-Rad protein assay using BSA as a standard.

Histochemical Staining for GUS

Tissues were stained with 1 mM X-gluc (5-bromo-4-chloro-3-indolyl β-glucuronide) staining solution containing 50 mM $NaPO_4$, pH 7, 0.05% Triton X-100, and 0.1% β-mercaptoethanol for 12 to 16 h at 37°C. Tissues were then fixed in 50% ethanol, 5% glacial acetic acid, and 10% formaldehyde for 2 h. Chlorophyll was removed by extensive washing of the tissue in 70% ethanol. Tissues for embedding were subsequently dehydrated though solutions of increasing ethanol concentration (twice at 70% for 8 h; once at 80% for 30 min; once at 90% for 30 min; and once at 100% for 30 min). After thorough dehydration samples

were cleared in four changes of xylene, two changes of paraffin-saturated xylene, and four changes of molten paraffin. Embedded samples were then sectioned to 12 to 16 μm of thickness. Sections were attached to glass slides, dried, deparaffinized with xylene, and mounted with acrylic resin.

RESULTS

Induction of Sesquiterpene Cyclase Gene Expression by Cryptogein

Cryptogein, a fungal protein known to induce necrosis in tobacco plants (Billard et al., 1988; Ricci et al., 1989) and capsidiol accumulation in tobacco cell cultures (Blein et al., 1991; Milat et al., 1991), was used as an elicitor in the following experiments. Cryptogein at 2 to 10 nM was sufficient to induce sesquiterpene cyclase genes in tobacco cell cultures within 4 h (data not shown). When infiltrated into tobacco leaves at nanomolar concentrations (10–100 nM), cryptogein caused necrotic symptoms (Fig. 1). The hypersensitive response-like necrosis was evident in the infiltration zones within 12 to 18 h of infiltration. Unlike a previous report, in which cryptogein was supplied through cut petioles and migrated throughout the plants, causing systemic necrosis (Devergne et al., 1992), the localized infusion of cryptogein induced hypersensitive response-like necrosis that was restricted to the infiltration zones; no necrosis was observed in distal tissues even after several days.

Figure 1. Elicitin-induced hypersensitive response in tobacco. Zones left of the midvein were infiltrated with approximately 25 μL of 50 nM cryptogein, whereas the comparable zones right of the midvein were infiltrated with water. The leaf was photographed 2 d after infiltration.

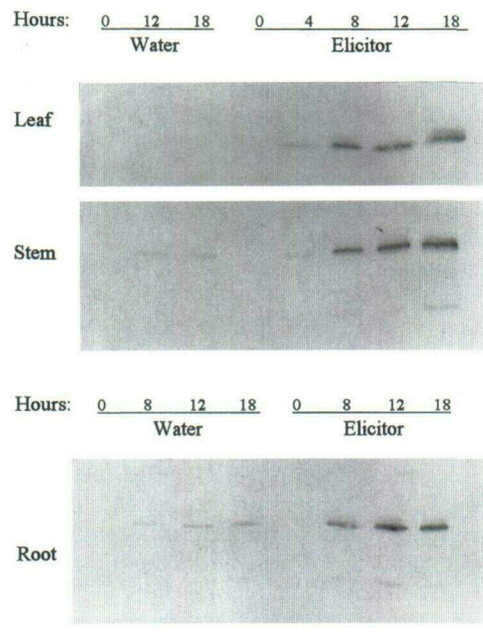

Figure 2. Induction time course of sesquiterpene cyclase protein in elicitin-treated plant tissues. Leaf tissues were infiltrated with either 50 nM cryptogein or water, whereas cut segments of stems and roots were incubated in water or 50 nM cryptogein. Equal quantities of protein (25 μg per lane) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed for the cyclase protein using a monoclonal antibody (Vögeli et al., 1990). The immunodetected protein is approximately 61 kD, and the small, minor peptide detected at the later times of elicitor treatment is approximately 25 kD.

To effectively interpret the behavior of the EAS4 promoter-GUS gene fusions in transgenic plants, the time course of cyclase protein accumulation in nontransgenic tobacco was determined. Cyclase protein was not detectable in untreated tobacco leaves, roots, or stems (time zero), but accumulated to high levels upon cryptogein treatment (Fig. 2). Detectable levels of cyclase protein were induced in less than 4 h, with a maximum occurring 12 to 18 h after elicitor treatment. The timing of cyclase accumulation in tobacco leaves was correlated with the development of the necrosis symptoms caused by cryptogein. Small amounts of cyclase protein accumulated in water-treated root and stem segments but not in water-infiltrated leaves maintained on whole plants, indicating a wound-inducible component of the sesquiterpene cyclase in these tissues.

Elicitor- and Pathogen-Inducible Activation of the EAS4 Promoter

The DNA sequence representing 1.2 kb of the EAS4 promoter (−1148 to +67) was fused in frame with the *GUS* reporter gene in the binary vector pBI101.1 and was used to generate transgenic tobacco plants. Of 12 independent transgenic lines tested, 1 had no GUS expression and the remaining 11 showed strong inducibility of GUS activity in leaves challenged with elicitor (discussed more fully below). As shown in Figure 3, the temporal induction of the

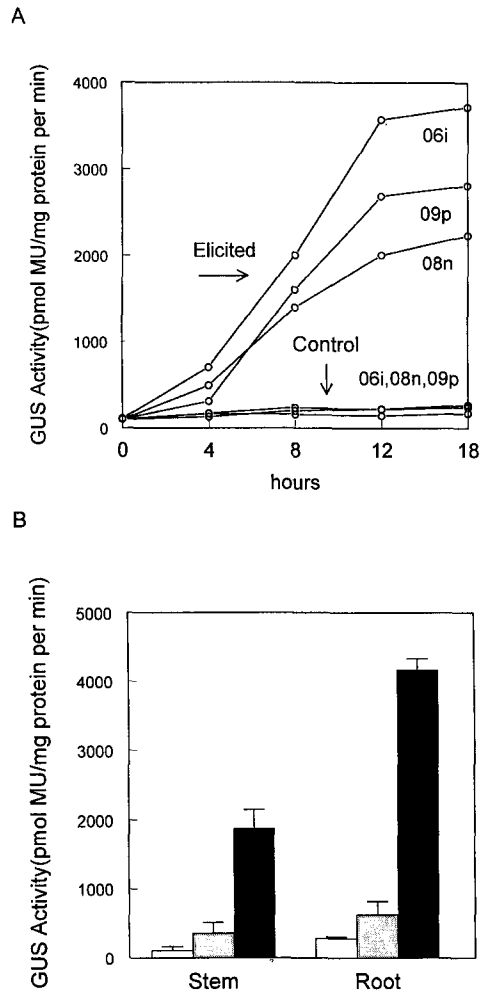

Figure 3. Expression of the EAS4 promoter-GUS gene construct in transgenic plants. A, Time course of elicitin-inducible expression of GUS activity in leaves infiltrated with water or 25 nM cryptogein. 06i, 08n, and 09p represent three independent transgenic lines of tobacco containing the EAS4-GUS construct. The data represent the average for two separate experiments with four individual plants used per time point. B, Elicitin-inducible expression of GUS activity in stem and root segments incubated in water or 100 nM cryptogein. Samples were collected immediately after preparation of the tissue segments (open bars), and after a further incubation of 18 h in water (shaded bars) or elicitin (black bars). The values indicated are averages of GUS activity for two independent transgenic lines (03-F and 09-P) with four individual plants used per determination. MU, 4-Methyl umbelliferone.

EAS4 promoter closely resembled that of endogenous sesquiterpene cyclase genes (compare with Fig. 2). No significant increase in GUS activity was observed over the time course of the water treatment (control). To determine if the elicitor-inducible GUS activity was specific for the EAS4 promoter, transgenic plants containing the CaMV 35S promoter-GUS gene fusion were also tested. No increase of GUS activity in any of the CaMV 35S-GUS transgenic leaves treated with elicitor was observed (data not shown).

To determine if the EAS4 promoter was functional in tissues other than leaves, roots and stems from transgenic tobacco plants were segmented and incubated in water or 100 nM elicitin. Figure 3B shows that elicitor treatment resulted in an approximately 15-fold increase in GUS activity in both stems and roots compared with the control. Similar to the wound-induction pattern of the endogenous sesquiterpene cyclase protein, a small increase in GUS activity was also observed for water-treated stem and root segments.

Although the accumulation of sesquiterpene cyclase was not detectable in healthy tobacco tissues by immunoblot analysis, transgenic tobacco containing the EAS4 promoter-GUS gene fusion showed varying levels of basal expression of GUS activity in leaves, stems, and roots. This may be due to some leakiness of the EAS4 promoter, a greater sensitivity of the GUS assay, enhanced stability of the GUS mRNA and protein, absence of a negative (suppressor) element within the 1.2-kb EAS4 promoter, and/or false positive measurements of GUS activity. Regardless of the reason, low levels of GUS activity were observed in a number of the transgenic lines.

Infiltration of transgenic tobacco leaves with an incompatible isolate of *P. syringae* pv *syringae* 61 resulted in a 5.4-fold induction of GUS activity within 12 h (Table I). In addition, infiltration of transgenic tobacco leaves with *P. syringae* pv *syringae* 61 *hrpH*, a mutant incapable of inducing a hypersensitive response (Huang et al., 1992), also resulted in a significant increase in GUS activity (Table I).

To determine if the EAS4 promoter was inducible by fungal pathogens, detached leaves of transgenic tobacco were inoculated with *P. parasitica* var *nicotianae*, and the infected leaf tissue was analyzed for GUS activity after 1 d of incubation, a relatively early stage in disease development. Although it has been reported that race 0 and race 1 of *P. parasitica* cause different disease symptoms on different tobacco cultivars (Tedford et al., 1990), no significant difference in visual symptoms caused by these two races was evident on detached cv Xanthi leaves. Consistent with this observation, race 0 and race 1 of *P. parasitica* induced expression of the EAS4-GUS gene fusion equally well (Table I).

Table I. *Induction of EAS4 promoter in a representative transgenic tobacco line (line 09-P) by infection with P. syringae pv syringae 61 (P.s.s. 61), the P. syringae pv syringae 61 hrpH mutant, and P. parasitica var. nicotianae (P.p.) race 0 and 1*

Infection	GUS Activity[a]	Induction
	pmol MU[b] mg^{-1} protein min^{-1}	*-fold*
Control	128	1.0
P.s.s. 61	685	5.4
P.s.s. 61 hrpH mutant	365	2.9
Control	136	1.0
P.p. race 0	1003	7.4
P.p. race 1	897	6.6

[a] No measurable GUS activity was detected in the free-living *Pseudomonas* or *Phytophthora* organisms. [b] MU, 4-Methyl umbelliferone.

Spatial Expression Pattern of the EAS4-GUS Gene Fusion

To examine cell- and tissue-specific expression of the EAS4 promoter, histochemical analysis of GUS activity in transgenic tobacco was carried out. The data presented in Figure 4 are representative of a series of observations made at different times with several independent lines containing the EAS4-GUS gene fusion. As shown in Figure 4A, elicitor-inducible GUS activity was restricted to the infiltration zones. The inducibility of the EAS4 promoter was apparent in all cell types across a leaf cross-section, but the staining of leaf epidermal cells was relatively weak (Fig. 4B). This is in marked contrast to the elicitor-induced GUS expression in stems and roots.

Intense GUS staining was observed in the subepidermis and trichomes in both stem and root sections (Fig. 4, C–L). In stems pronounced GUS activity was confined to the periderm, cambium, phloem, and primary xylem tissues. Very little activity was observed in the cortex or pith regions after elicitor treatment (Fig. 4, C–F). Strong elicitor-inducible EAS4-GUS expression was also associated with specific cell types in roots (Fig. 4, G–L). Control histochemical stained root segments (transgenic but incubated with water only) exhibited very limited, if any, staining for GUS activity (Fig. 4, G, I, and K), whereas peridermal cells and vascular cambium exhibited intense GUS staining after elicitor treatment (Fig. 4, H, J, and L).

Effects of SA, MJ, and H_2O_2 on Expression of the EAS4-GUS Gene Fusion

SA has been implicated as a key signal molecule involved in the establishment of SAR (Malamy et al., 1990; Metraux et al., 1990; Gaffney, et al., 1993). Recently, evidence that SA plays an important role in the expression of local resistance has also been reported (Delaney et al., 1994; Ryals et al., 1995). To determine if SA has any effects on the induction of sesquiterpene cyclase genes, GUS activity and sesquiterpene cyclase enzyme activity in leaf discs from transgenic and wild-type plants treated with increasing concentrations of SA were determined. Figure 5 shows that SA treatment resulted in a very modest increase in both GUS and cyclase activities over the water treatment.

MJ, a lipoxygenase product of linolenic acid, has also been proposed to be a signal molecule that is released by plants in response to various stimuli, including wounding or pathogen attack (Gundlach et al., 1992; Hildmann et al., 1992; Reinbothe et al., 1994). Although sesquiterpene cyclase activity is slightly wound inducible, MJ treatment had

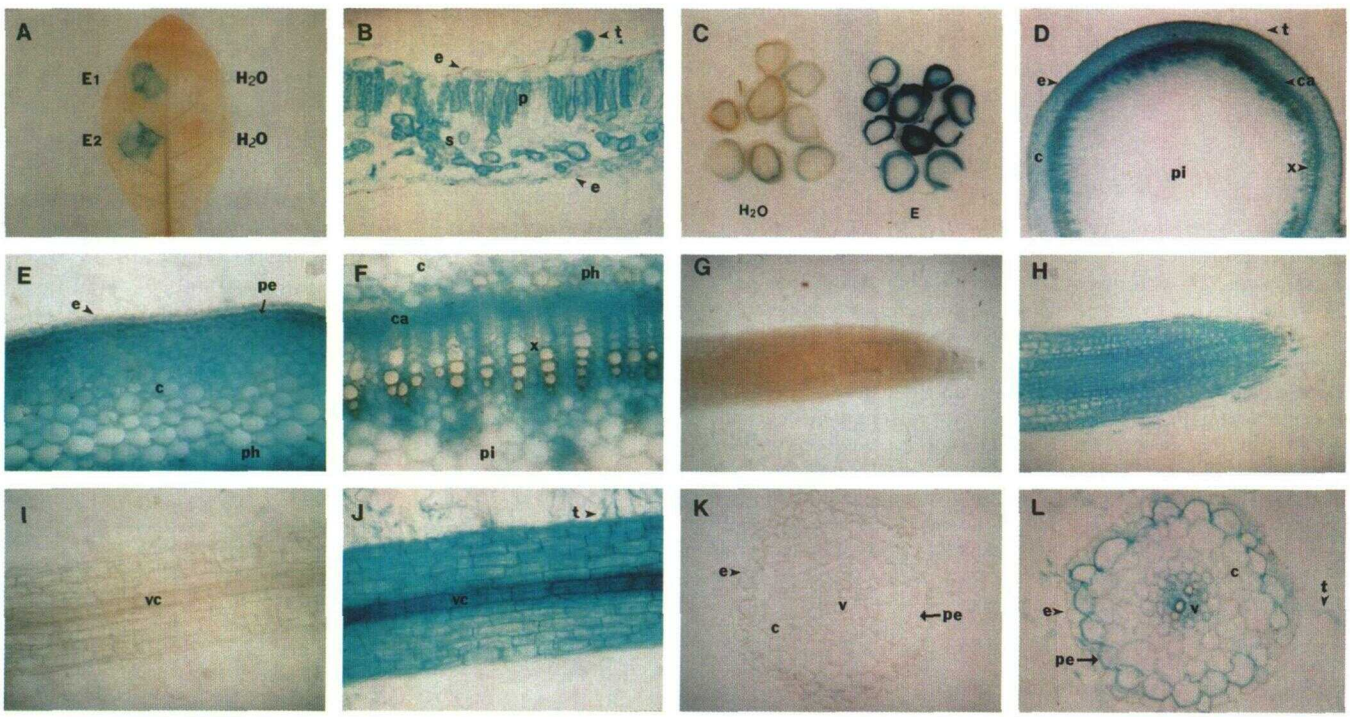

Figure 4. Histochemical localization of GUS expression in transgenic plants containing the *EAS4-GUS* gene construct. Leaves infiltrated with water or 25 nM cryptogein were incubated 8 h before staining for GUS activity. Root and stem segments were incubated for 12 h in water or 100 nM cryptogein before staining for GUS activity. A, GUS expression in a leaf infiltrated with elicitin (E1, E2) or water; B, cross-section of a leaf area infiltrated with elicitin (X75); C through F, cross-sections of a stem segment treated with elicitin (X2, X15, X60, and X60, respectively); G, a root tip of an *EAS4-GUS* transgenic plant incubated in water; H, longitudinal section of a root tip after incubation with elicitin (X60); I, root segment of an *EAS4-GUS* transgenic plant incubated in water (X75); J, root segment after incubation with elicitin (X75); K, cross-section of a root segment from an *EAS4-GUS* transgenic plant incubated in water (X95); and L, cross-section of a root segment treated with elicitin (X95). C, Cortex; ca, cambium; e, epidermis; p, palisade parenchyma; pe, periderm; ph, phloem; pi, pith parenchyma; s, spongy parenchyma; t, trichome; x, xylem; and vc, vascular tissue.

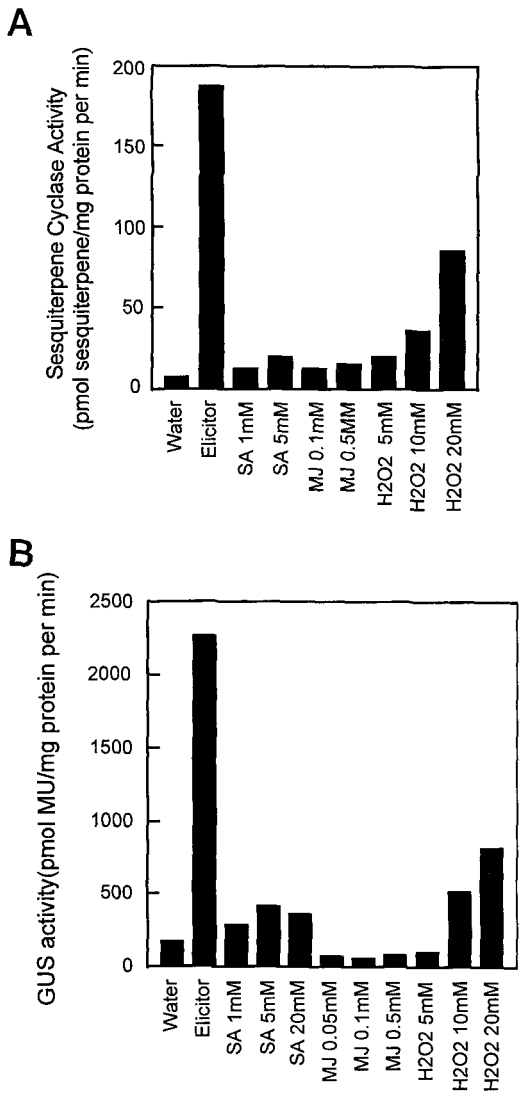

Figure 5. Effects of SA, MJ, and H_2O_2 on the induction of the EAS4 promoter and endogenous sesquiterpene cyclase activity. Leaf discs from control and transgenic plants containing the *EAS4-GUS* gene fusion (line 09-P) were incubated with water or the indicated concentrations of SA, MJ, or H_2O_2 for 24 h. Leaf tissue 12 h after infiltration with 25 nM cryptogein was used for comparison. Sesquiterpene cyclase activity (A) was determined for leaf discs from non-transgenic plants, whereas GUS activity (B) was determined for leaf discs from *EAS4-GUS* transgenic plants. MU, 4-Methyl umbelliferone.

a limited effect on cyclase activity. In fact, MJ treatment inhibited the expression of the EAS4 promoter-GUS gene fusion in tobacco (Fig. 5). GUS activity after MJ treatment was only 25 to 40% that of the water controls. However, a slight discrepancy between quantitative levels of GUS activity and cyclase activity for MJ treatment was noted, and the small increase in cyclase activity by MJ treatment may represent the expression of cyclase genes other than *EAS4*.

Elicitor treatment or attempted infection with an avirulent pathogen strain can cause the rapid production of H_2O_2 (Mehdy, 1994; Tenhaken et al., 1995), which has been demonstrated to induce defense gene expression as well as programmed cell death (Levine et al., 1994; Tenhaken et al., 1995). Since phytoalexin accumulation is usually associated with these responses, the effects of H_2O_2 on the induction of the EAS4 promoter and endogenous sesquiterpene cyclase were examined. As shown in Figure 5, a moderately high concentration of exogenously applied H_2O_2 (5 mM) had little effect on the induction of either GUS or cyclase activities, but 20 mM H_2O_2 induced a small increase in both cyclase and GUS activity.

Effect of 5′ Deletions on Elicitor Inducibility of the EAS4 Promoter

The DNA sequence of the EAS4 promoter (Fig. 6) was compared with several other defense gene promoters, such as those for PAL (Lois et al., 1989), CHS (Harrison et al., 1991), IFR (Oommen et al., 1994), PR1 (Uknes et al., 1993), and PR2 (van de Locht et al., 1990), for putative regulatory motifs, and other sequences. The EAS4 promoter does not contain any sequences similar to the P-box (CCANCNA-ACNCC) or H-box (CCTACCN$_7$CT), *cis*-elements that have been found in several PAL and CHS promoters and implicated in elicitor inducibility (Lois et al., 1989; Yu et al., 1993; Dixon and Paiva, 1995; Hahlbrock et al., 1995), but does contain sequences that are similar to the G-box (−366) (Weisshaar et al., 1991; Schindler et al., 1992; Williams et al., 1992), the as-1 (−195) (Katagiri et al., 1989; Lam et al., 1989), and the myb-box (−94) (Biedenkapp et al., 1988; Baranowskij et al., 1994). The EAS4 promoter does not contain an ethylene-responsive GCC-box, which is conserved in a number of tobacco pathogenesis-related protein genes (Ohme and Shinshi, 1990; Eyal et al., 1993; Hart et al., 1993; Shinshi et al., 1995).

The EAS4 promoter sequence was also compared with the EAS3 promoter, a cyclase promoter that may have only very limited transcriptional activity. No cDNA having 5′ or 3′ nontranslated sequences corresponding to those in *EAS3* has yet to be isolated, and transient expression experiments using EAS3 promoter-GUS fusions (presented below) suggest that this promoter is not elicitor inducible. Other than identical sequences from −63 to the translation start site, there are no other similarities between these two promoters.

To functionally map *cis*-elements that contribute to the elicitor-inducible nature of the EAS4 promoter, a number of 5′ deletions in the EAS4 promoter were fused to the *GUS* reporter gene and introduced into tobacco plants (Fig. 7). Approximately 12 independent transformants for each construct were generated and at least 5 individual plants from the R_1 generation for each transformant were tested. Throughout this analysis fully expanded, healthy leaves (7th and 8th from the bottom) from 2-month-old plants were used. Variance in the levels of GUS expression for each transgenic line was observed and was generally within 50% of the average value. This level of variation might be expected to some extent, given that the transgenic plants represented a segregating population homozygous/heterozygous for the transgene.

A

```
-489  CCGCGATTGGAGGATGTTGTACGTCGAGCTACGCGGCACCGCGCTTAATA
      TTTACTCGGTCAAGAAGGAACGGGGATGGTGGTCAACGAAACACGACGGG
-389  CCCGACATCATGCCTGACAACCCGCCGTGGGTGAAGAAGTCGACGTTGGA
      AAAGAGCTACAGCCTGCTCCACGCGGATGCGGGGATGGCCGCTGACTACA
-289  GAAAGTGCGTTTCCCGCCACCCGGGGCGAGCCCGGGTTTTGAAGATCAAT
      GCTGACCGAACCAGACGGCGGTACGTCATCCGCTTGAGGGTAGAGACGGA
-189  TCAGTTCTTGTTGTCGTGTGTCGAACTCGGGACGTTTGTCACATGGCTGG
      ACGGGTTATTCGCCGCCATCAACGTGTCGCCGCCAATCGACGAGCGCGAC
-89   TTTCCCAGAGACTTTAGCGTGCCACGGATCAATTACATTAACTAGTCTCT
      CACCACTATATATACTTGTCCCTTCTCTTCCATTTAAGTAGAGTTCCTTT
                                  +1
+12   CTTTCTTCCTTAAAACTTAAAAGAACAAGTAAAAATACACTCATCTTTAA
+62   TTAGCA
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B

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-1148 AAGCTTTACGAATTAGATGTAAAAAGACACAAACTACTTATATATATTAC
      CAAAGTAACTTGAAAGTTTAAAATTTCAATTAGAACTATAGTAGGGTAAA
-1048 ACTGTCTATTTAAAATCAGTATTTAAAAAGGCATGAGCGAAAGATGAGGC
      GTTTTATCTAACACGAAGCGAGGTGTAAGCCCCATGGTGTTTTATTTTTA
- 948 TATTTTATAAATTTATAAAATCATTATATAAATCAGAAAAATACACTAAA
      ATTGTGAAAAGTTAAAGAAAATTATAGAATTAATATATATATATATATAT
- 848 ATATATATATATATATATATATATATATATATATATAAATGTATGTGT
      GTGTGTGTGTGTATCGCATGCGCGCGACCATGCAACTTTTTTTTCTTGAA
- 748 AAAATAAAAGGCGTAAAGATACATTATACCTATGTCATCAAAACAATATA
      AAAACTAGAGCGATACCAAAGGAAATTTTAAATTCAAAAACTAACTTGAA
- 648 ATTAATATATTTAAAATTTCATTTTTTTTTGTGTGGAGAAAACAAAGCAT
      AACACTTTGCTTTGTAACACTTTGCCTAGGTGAATGTCAGGGCTTATGCT
- 548 CCACGATACTTATGCCCTGCCAGTACACCTCGCAGTGGGACTCGCTGAAA
      AAACGTCTTTGTTGTGAGAAATTGCAATTTTGAACCTCTACAATTTCGAC
- 448 AAAACCTTGGTTCGTGAAAACTGTTTGATTAACTTTTAGACCATCCAGTC
                                  G-box-like
      AATTTAACTCTAAACTGACCTAAATAAATACTACGTACACTAGTCTTTAA
- 348 GTTCATCAAAGTGGACTCTGCATTAATAATTGAAATTTATGCCGCAACAA
      TGACATTAGGTTTTATAAATAAAGTAATAGGAATTTGATAGTTCCAGGAA
- 248 ACAACTCTACAGTACTCCCTTATTTTGTGCCTTTTTAAATAATATTATTC
          as-1
      AGTTGACGAAACAAATAAATAAAATATTTGGGAAACTGGATCAATAGACC
- 148 CCAGACGCCAACAATGAATCAAAAGGCTGCTAGCTAGTGTAAAGTCTAGT
          myb
      AAGGCAACTGGGAAATTAAATGATTAGGTGCTTTTGATCAATTACATTAA
                                                  +1
-  48 CTAGTCTCTCACCACTATATATACTTGTCCCTTCTCTTCCATTTAAGTAG
+   3 AGTTCCTTTCTTTCTTCCTTAAAACTTAAAAGAACAAGTAAAAATACACT
+  53 CATCTTTAATTAGCA
```

Figure 6. DNA sequences for the EAS3 (A) and EAS4 (B) promoters. The transcription initiation sites are designated as +1 based on primer-extension analysis for the EAS4 promoter (Zhu et al., 1995). Possible CAAT and TATA sequences are found at −64 and −33 relative to the transcription start site and are indicated by bold lettering. The sequences from −63 to +67 are identical in both *EAS3* and *EAS4* and are underlined. Sequences having similarity to the G-box (Weisshaar et al., 1991; Schindler et al., 1992; Williams et al., 1992), as-1 (Katagiri et al., 1989; Lam et al., 1989), and myb (Biedenkapp et al., 1988; Baranowskij et al., 1994) recognition sequences are underlined and annotated.

Figure 7 shows the range for control and elicitor-induced GUS activity levels for all independent transgenic lines harboring the full-length EAS4 promoter or the 5′ deletion constructs as indicated. The levels of GUS expression for each deletion construct were then averaged and are summarized in Table II. The variation seen within the various transgenic lines is consistent with reports from other studies (Peach and Velten, 1991; Uknes et al., 1993) and can be attributed to positional effects, how the different surrounding genomic DNA influences expression of the transgene in the independent transformants, and variations in the number of transgenes integrated in the plant genome. Although kanamycin-segregation analysis did demonstrate that several transgenic lines harbored multiple copies of the transferred DNA (data not shown), no correlation between gene copy number and expression was obvious.

Deletion of the EAS4 promoter to −567 decreased the inducible GUS activity level to 50% of that in plants with the full-length promoter (−1148 to +67) (Table II). Deletion to −212 decreased inducible GUS activity by an average of 90%. Further deletion to −160 decreased GUS activity to 2% of that in plants with the full-length promoter. Although deletion to −160 removed DNA elements important for high levels of GUS expression, the sequences downstream of −160 were sufficient to direct elicitor-inducible expression in leaf, stem, and root tissues (stem and root data not shown). As shown in Figure 7, a 12- to 20-fold induction of GUS activity in leaves was observed for all transgenic lines examined.

Further deletion of the promoter to −115 and to −63, which contains a consensus TATA box sequence, completely abolished elicitor-inducible expression of the chimeric gene. No increase in basal expression of GUS activity was observed for either the −115 or the −63 deletions (Fig. 7), which would have implied the presence of a *cis*-sequence imposing suppression of gene expression. Of 12 lines containing the −115 construct, 1 showed extraordinarily high levels of constitutive GUS expression, which may reflect a unique integration site of this transgene into the genome.

Similar results were obtained using transient expression analysis. The 5′ deletion constructs cloned into pBI221 vectors were introduced into tobacco protoplasts by electroporation and analyzed for GUS activity after incubation of the protoplasts with and without additional elicitor (Fig. 8). As observed with the stable transgenic lines, deletions into the EAS4 promoter brought about a quantitative decrease in GUS expression. Moreover, in spite of the high background of GUS expression that was presumably due to the elicitation occurring during the preparation of the protoplasts (Roby et al., 1991; Mohan et al., 1993), the protoplasts were able to respond to a subsequent elicitor treatment. The levels of GUS activity for 5′ deletions to −567, −212, and −160 were significantly elevated by the elicitor treatment and were approximately 2-fold greater than the GUS activity measured in protoplasts not receiving additional elicitor. In contrast, elicitor treatment resulted in only a marginal increase in GUS activity for the −63 deletion, similar to the increase in GUS activity observed for a promoterless GUS construct. For comparison, neither of the

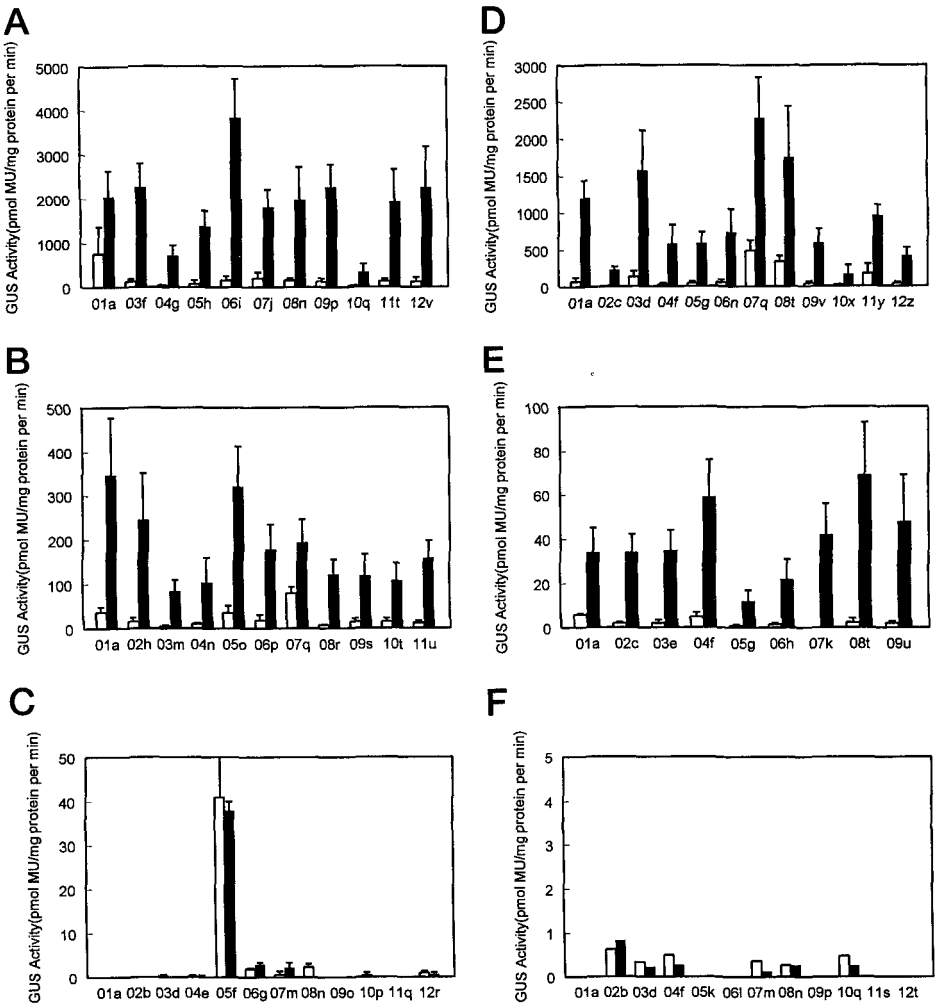


Figure 7. Deletion analysis of the EAS4 promoter. Constructs were prepared by deleting 5′ sequences from the full-length (−1148 bp) EAS4 promoter-GUS construct (A); the 5′ deletion end point coordinates are −212 (B), −115 (C), −567 (D), −160 (E), and −63 (F) relative to the transcription start site (+1). GUS activity in leaf samples of 9 to 12 independent transgenic lines for each deletion construct was determined 12 h after infiltration with water (control, open bars) or 25 nM cryptogein (elicited, black bars). Values in the bar graphs represent the means and SEs for five to eight separate experiments. MU, 4-Methyl umbelliferone.

constructs using the EAS3 promoter fused to GUS, and the promoterless GUS construct exhibited significant elicitor-inducible GUS activity.

Gain-of-Function Analysis of Sequences Upstream of −87

The 5′ deletion studies suggested that sequences from −160 to −115 were critical for elicitor inducibility. To more definitively identify those *cis*-elements responsible for the elicitor inducibility, several gain-of-function constructs were tested in transgenic plants (Fig. 9). Somewhat unexpectedly, the sequences upstream of −87 were not sufficient to confer elicitor inducibility onto the CaMV 35S minimal promoter (−90 to +8). Only two of five independent transformants harboring the EAS4 promoter sequence between −160 and −87 showed small increases in GUS activity after elicitor treatment. In addition, the variance in the GUS activity between independent lines for these constructs was much greater than for the 5′ deletion constructs. For example, GUS activity levels in independent transformants containing the −160 to −87 construct varied from 14 to 209 units for control and from 15 to 291 units for elicitor treatment. Nonetheless, none of the gain-of-function constructs demonstrated a convincing level of elicitor inducibility.

DISCUSSION

Capsidiol, the principal sesquiterpene phytoalexin found in tobacco and other Solanaceous plants, is not detectable in healthy (control) plant tissues but rapidly accumulates in tobacco plants challenged with viral, bacterial, or fungal pathogens (Bailey et al., 1975; Guedes et al., 1982; Ricci et al., 1989) and in cell cultures treated with a variety of elicitors (Brooks et al., 1986; Vögeli and Chappell, 1988; Whitehead et al., 1989; Milat et al., 1991). Work from this

Table II. *Effect of 5′ deletions of the EAS4 promoter on the average levels of GUS expression in transgenic tobacco leaves*

5′ Deletions	Average GUS Activity[a]		Average Induction	No. of Lines[b]
	Control	Elicited		
	pmol MU[c] mg^{-1} protein min^{-1}		*-fold*	
−1148	173 ± 201	1889 ± 908	10.9	11
−567	120 ± 148	921 ± 665	7.7	12
−212	23 ± 22	187 ± 89	8.1	11
−160	2 ± 2	34 ± 18	17.0	9
−115	4 ± 12	4 ± 11	1.0	8
−63	0.2 ± 0.2	0.2 ± 0.2	1.0	6

[a] Average GUS activity represents the sum of the average activities for each transgenic line within a deletion series divided by the number of lines. [b] Transgenic lines not having any GUS expression, control or inducible, were excluded from these calculations (see Fig. 7). [c] MU, 4-Methyl umbelliferone.

laboratory (Vögeli and Chappell, 1988; Vögeli et al., 1990) and others (Zook and Ku'c, 1991) has correlated the accumulation of this and related sesquiterpene phytoalexins with the induction of key enzymes in the isoprenoid biosynthetic pathway. In particular, EAS enzyme activity, a sesquiterpene cyclase and putative branch point enzyme regulating the diversion of C from the central isoprenoid pathway toward sesquiterpene biosynthesis, was absent from control cells and was rapidly induced in response to elicitor treatment (Vögeli and Chappell, 1988). The induction of this enzyme activity was further correlated with the de novo synthesis of the enzyme protein (Vögeli et al., 1990), an induction in the steady-state levels of the EAS mRNA (Facchini and Chappell, 1992), and a rapid increase in the transcription rate of the EAS gene(s) (Vögeli and Chappell, 1990; Facchini and Chappell, 1992).

Altogether, this evidence suggested that, at least in tobacco, phytoalexin biosynthesis and accumulation in response to pathogen challenge was regulated primarily by transcriptional control of key biosynthetic enzymes. However, although the evidence for this assertion was quite compelling, it was complicated by the observation of a 12- to 15-member *EAS* gene family, which raised several questions about how this family might be differentially regulated by different types of pathogens and/or elicitors.

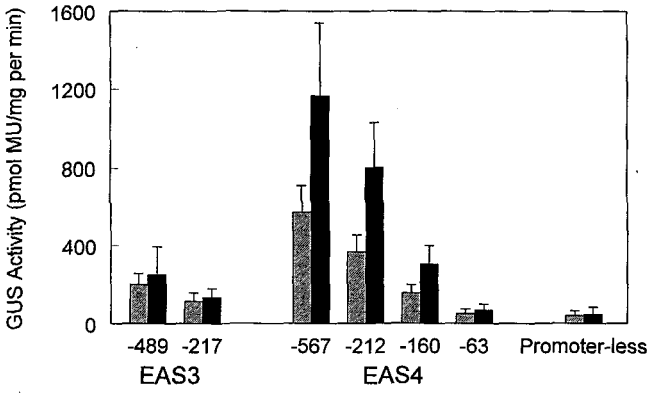
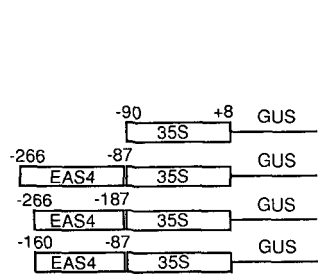


Figure 8. Transient expression of the EAS3-GUS and EAS4-GUS constructs. GUS constructs having 5′ promoter lengths relative to the transcription start site as indicated were electroporated into tobacco protoplasts. Shaded bars, Control; black bars, elicitor-treated. The protoplasts were subsequently incubated in the absence or presence of additional elicitor, cellulase (1 μg/mL), for 18 h before determining GUS enzyme activities. Values in the bar graph represent the means and SEs for four or more replicate experiments. MU, 4-Methyl umbelliferone.

Formally, each member of the *EAS* gene family could be differentially regulated such that one gene member might be expressed in leaves, another in stems, and yet another in roots. Although our data cannot exclude this possibility for other *EAS* gene members, when the EAS4 promoter is fused to the *GUS* reporter gene, this promoter confers an inducible expression pattern onto the reporter gene that is similar, if not identical, to that observed for cyclase expression throughout the plant. For example, the time course of GUS activity in elicitor-infiltrated *EAS4-GUS* transgenic plants paralleled the pattern observed for the endogenous cyclase protein. Both the endogenous cyclase protein levels (Fig. 2) and GUS enzyme activity (Fig. 3A) reached half-maximal levels within 8 h and maximal levels by 12 to 18 h after elicitor infiltration into leaf samples. Likewise, the small but significant wound induction of cyclase activity (data not shown) and cyclase protein (Fig. 2) within 18 h of preparing stem and root segments was also apparent for GUS activity in stem and root segments prepared from the transgenic plants harboring the EAS4-GUS construct (Fig. 3B).

Earlier studies of elicitor-treated cell cultures suggested that accumulation of sesquiterpene phytoalexins in pathogen-challenged tobacco tissues (Bailey et al., 1975; Guedes et al., 1982; Ricci et al., 1989) was dependent on an induction of sesquiterpene cyclase enzyme activity (Vögeli and Chappell, 1990; Facchini and Chappell, 1992). Furthermore, due to the complex nature of plant-pathogen interactions and the possibility that different pathogens might induce different signal transduction pathways leading to phytoalexin accumulation, select members of the cyclase gene family might be expected to respond differentially to different classes of pathogens. The results presented in Table I demonstrate that this is not the case for the *EAS4* gene. GUS expression was strongly induced in response to bacterial or fungal inoculations of the *EAS4-GUS* transgenic plants. A similar activation of GUS expression in response to viral challenge has been observed (data not shown), although such data are complicated by the very



	GUS Activity (Control/Elicited) for independent lines				
	1	2	3	4	5
35S	66/74	74/73	20/30	27/94	19/17
EAS4 35S	11/13	22/15	517/602	138/201	99/91
EAS4 35S	35/41	59/50	372/374	24/22	-
EAS4 35S	20/19	209/291	14/15	92/87	72/169

Figure 9. Gain-of-function analysis of EAS4 promoter fragments. EAS4 promoter fragments with 5′ and 3′ coordinates as indicated were ligated to a minimal CaMV 35S promoter (−90 to +8) (reference), and these chimeric promoter fragments were fused to the *GUS* gene. GUS activity in leaf samples of four or five independent transgenic lines for each deletion construct was determined 12 h after infiltration with water (control) or 25 nM cryptogein (elicited). Values represent the average GUS activity determined for two to five individual plants for each transgenic line.

strong wound response that occurs when rubbing the leaf tissue with carborundum, a surface abrasive often used when inoculating with viral pathogens.

Histochemical analysis demonstrated that the 1.2-kb EAS4 promoter exhibits a precise spatial expression pattern in response to elicitor treatment, many facets of which are consistent with *EAS* gene expression representing a plant defense response. Many pathogens infect host plants through wound sites in the root system or through weather-induced breaks in the epidermal layer of stem cells. Once the pathogens have penetrated into the plant tissue, they spread throughout the plant via the vascular system (Agrios, 1988). The strong induction of the EAS4 promoter in root and stem subepidermal cells and in the cells immediately surrounding the vascular tissues are consistent with the plant's need to generate an effective barrier to microbial ingress and prevention of systemic spread. Expression in epidermal cells would not necessarily be advantageous, since the epidermis is easily breached/wounded in many circumstances and may not represent the first host cells to interact with an invading pathogen.

Limited Induction of the EAS4 Promoter by SA, MJ, or H_2O_2

Inspection of the DNA sequence for the EAS4 promoter revealed the presence of a TGACG sequence located at −195. This sequence is similar to the as-1 motif found in the CaMV 35S promoter, a *cis*-acting element that has been correlated with root-specific expression (Lam et al., 1989). The as-1 sequence has also been implicated as an SA-responsive element and is present in the promoter sequences of several plant genes associated with the establishment of SAR (Qin et al., 1994). Although the function of this sequence in the EAS4 promoter is unknown, the EAS4 promoter and endogenous cyclase genes are only weakly induced by SA. Moreover, the onset of SAR is a relatively late event accompanied by the accumulation of SA (Malamy et al., 1990; Metraux et al., 1990; Ryals et al., 1995), whereas the induction of defense genes involved in phytoalexin biosynthesis (such as *EAS*) is very rapid (Baillieul et al., 1995). We cannot, however, rule out the possibility that low level activation of these defense genes occurs later in systemically protected tissues.

Similar to SA, MJ and its precursor JA reportedly trigger wound- and pathogen-induced defense response (Farmer and Ryan, 1992; Reinbothe et al., 1994), including the induction of many secondary and phytoalexin biosynthetic pathways (Gundlach et al., 1992; Mueller et al., 1993). Although the EAS4 promoter and endogenous cyclase genes were shown to be wound inducible, our data suggest that the wound inducibility does not involve MJ. MJ alone only slightly induced cyclase activity, but actually suppressed EAS4 promoter activity.

Choi et al. (1994) made a somewhat similar observation in elicitor-treated potato tuber slices. Exogenous MJ suppressed the ability of arachidonic acid to induce HMG2 mRNA, an mRNA for a specific HMGR isozyme correlated with sesquiterpene phytoalexin biosynthesis, as well as the accumulation of sesquiterpene phytoalexins in potato tuber slices. However, Choi et al. (1994) also observed that MJ induced the mRNA for another HMGR isozyme, HMG1, and steroid glycoalkaloid accumulation in wounded tuber slices. Ellard-Ivey and Douglas (1996) recently reported that MJ induced the expression of a number of furanocoumarin phytoalexin biosynthetic genes, including *PAL*, *4CL*, and *BMT* in parsley cell cultures. Therefore, the role of MJ in wounding and defense responses appears to be plant and gene specific and situation dependent.

H_2O_2 is another putative signal molecule purported to transduce elicitor-pathogen-recognition events into several defense responses, including the activation of defense gene expression (Mehdy, 1994). A low concentration of H_2O_2 induces expression of several cellular protectant genes, whereas a high concentration can induce cell death (Levine et al., 1994). In the current study sesquiterpene cyclase and the EAS4 promoter were inducible by high concentrations of exogenous H_2O_2 compared with elicitor treatment. The rather modest induction of the EAS4 promoter and endogenous cyclase enzyme activity by high concentrations of H_2O_2 may simply reflect a secondary effect associated with cellular damage caused by such high concentration of this activated O_2 species. Therefore, it is difficult to discern the physiological relevance of such high H_2O_2 concentrations on the induction of sesquiterpene cyclase gene expression at this time.

Organization of the EAS4 Promoter

In terms of *cis*-sequences regulating the EAS4 promoter, our results indicate that there are at least three elements that serve to enhance elicitor-inducible expression, each mediating an approximately 2- to 5-fold enhancement (summarized in Fig. 10). These quantitative elements are located from −160 to −1168 bp upstream from the transcription start site and are separated from *cis*-sequences that control pathogen/elicitor inducibility. The quantitative elements act singularly or in concert to enhance inducible expression greater than 50-fold, calculated as the difference between GUS activity driven by the full-length promoter and losses in expression brought about by sequential deletion of sequences to −160. The EAS4 quantitative elements also qualify as enhancer-like elements (Buratowski, 1995) because they promote elevated levels of expression when ligated to a minimal promoter. For example, a quantitative element supporting a 5- to 10-fold enhancement of control and elicitor-inducible expression was identified between coordinates −212 and −160 based on deletion analysis (Table II). Gain-of-function analysis with a fragment encompassing this element (−266 to −87) likewise enhanced expression from the minimal 35S CaMV promoter on average 2- to 4-fold.

The EAS promoter also contains multiple *cis*-sequences that control qualitative expression. An element controlling wound-inducible expression resides between −567 and −160, and loss of this sequence resulted in a 60-fold decrease in the level of control expression (Table II). Control values in these experiments actually correspond to the analysis of leaf tissue wounded due to the force of infiltrating water into its interstitial spaces. The level of GUS expression in leaf tissue not subject to the control water infiltration was considerably lower (data not shown).

Based on a combination of deletion, gain-of-function, and comparative analyses, we infer that two cooperating *cis*-sequences are essential for elicitor/pathogen inducibility. The −160 deletion mutant apparently retains all of the sequences necessary for elicitor/pathogen inducibility. Removal of an additional 45 bp to −115 abolished any responsiveness of the promoter to elicitor/pathogen treatment. However, the DNA sequence between −160 to −115 is not sufficient to confer elicitor inducibility. Gain-of-function constructs containing this sequence (either −266 to −87 or −160 to −87) fused to the minimal CaMV 35S promoter were also not responsive to elicitor (Fig. 9).

Sequence and functional comparisons between the EAS3 and EAS4 promoters (Figs. 3 and 8) support this contention. If sequences between −63 and +67 were sufficient for elicitor inducibility, then the −217 construct of EAS3 would be expected to have similar levels of activity as the −212 construct of EAS4. Therefore, the *cis*-sequence(s) between −160 to −115 must somehow cooperate with sequences between −87 and +67 of the EAS4 promoter, resulting in elicitor responsiveness. The necessity for the coupling of two distal *cis*-sequences, as suggested here, was recently demonstrated for several ABA-responsive genes by Shen et al. (1995), who referred to the interacting *cis*-elements as response complexes. It remains to be determined if we can identify an analogous elicitor-response complex within the EAS4 promoter.

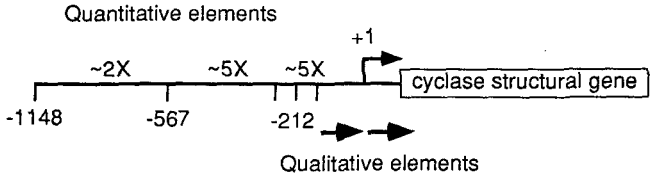


Figure 10. A schematic depiction of the EAS4 promoter indicating the relative positions of *cis*-sequences controlling quantitative and qualitative expression patterns. Quantitative elements refer to DNA sequences that when deleted reduce the overall level of expression by the factor shown. Qualitative elements refer to sequences that control elicitor inducibility, and their locations are inferred from a combination of loss-of-function and gain-of-function determinations.

Uniqueness of the EAS4 Promoter

An impressive number of elicitor-/pathogen-inducible genes from a wide range of plant species have been cataloged. Among these, promoters for the *CHS15* gene from bean (Dron, 1988; Harrison, 1991), the *PAL-1* (Lois et al., 1989) and *PR2* (van de Locht et al., 1990) genes from parsley, a *PR10a* gene from potato (Despres et al., 1995), an *IFR* gene from alfalfa (Oommen et al., 1994), and the *str246c* (Gough et al., 1995) and several *PR* (Uknes et al., 1993; Eyal et al., 1993; Shinshi et al., 1995) genes from tobacco have been examined in detail for *cis*-sequences controlling gene expression patterns. The CHS15 promoter, for example, contains G- and H-box elements that have been correlated with defense gene expression as well as proper developmental expression of the *CHS* gene in flowers and root tissues. A P-box element initially identified by in vivo footprinting (Lois et al., 1989) and subsequent isolation of BPF-1, a P-box-binding protein (da Costa e Silva et al., 1993), is conserved in sequence and position within several *PAL* genes in parsley (Hahlbrock et al., 1995). Although no sequence-specific motifs have yet been specified within the alfalfa IFR promoter, 400 bp of the TATA-proximal portion of this promoter was sufficient to direct proper developmental and elicitor-inducible expression of the *GUS* gene in transgenic alfalfa (Oommen et al., 1994).

The EAS4 promoter differs in many respects from these other well-characterized gene promoters. Although an overall organization of plant promoters with quantitative elements distal to the TATA box and qualitative elements more proximal to the transcription start site is conserved, sequence comparisons between these promoters with the EAS4 promoter have ruled out the conservation of any particular DNA motif or *cis*-sequence pivotal for elicitor/pathogen inducibility. Except for the EAS4 promoter, all of the defense genes analyzed to date exhibit some reactivity to the potentially pathogen-induced signal molecules SA, MJ, and H_2O_2. *GST* is induced by H_2O_2 (Levine et al., 1994), *PAL* and other phenylpropanoid biosynthetic genes are activated by MJ (Ellard-Ivey and Douglas, 1996), and many of the *PR* genes are defined by their SA inducibility (Ward et al., 1991; Ryals et al., 1994). Except for the EAS4 pro-

moter, all of the defense gene promoters analyzed to date exhibit developmental or constitutive expression in at least some tissues or cell types. The IFR promoter directs strong expression in root meristem, cortex, and nodules of transgenic alfalfa plants (Oommen et al., 1994), whereas the str246c promoter directs very high levels of root and sepal expression in transgenic plants (Gough et al., 1995). Except for a low level of wound-inducible expression, we have not detected any EAS4 promoter-directed GUS expression in any plant tissues, including flowers and all reproductive structures (S. Yin and J. Chappell, unpublished data), throughout plant development other than in those challenged with elicitors or pathogens.

De Wit (1992) and Oommen et al. (1994) have suggested that a pathogen-specific promoter might be useful to engineer novel disease resistance traits in plants. This type of strategy is predicated on the assumption that both an invading pathogen and a few host cells can be killed by the localized production of a highly toxic product. However, testing of this hypothesis is dependent on the availability and reliability of a promoter that drives expression of the cytotoxic product only in the context of pathogen challenge and not under any other circumstances. Given this rigorous criteria for expression specificity, the EAS4 promoter described here might be a suitable promoter for testing this hypothesis.

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